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(54) Title: ALTERATION OF PLANT AND PLANT CELL GROWTH CHARACTERISTICS

(57) Abstract

Plants with altered stature and other phenotypic effects, the most advantageous of these being precocious flowering and increased numbers of flowers, are produced by transformation of the plant genome with the cdc25 gene or a functional homologue thereof. The preferred source of such genes is the fission yeast *Schizosaccharomyces pombe*.

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ALTERATION OF PLANT AND PLANT CELL GROWTH CHARACTERISTICS

This invention relates to plants and plant cells with altered growth characteristics and to a method for producing such alterations. More particularly, but not exclusively, the invention relates to plants which exhibit precocious flowering.

In the fission yeast Schizosaccharomyces pombe, timing of and hence, cell size at mitosis is determined by expression of the cdc25 and the nim1 inducer genes and of the inhibitor gene wee1, which between them regulate the M-phase protein kinase $p34^{cdc2}$ [Nature, Vol.344, pages 549 to 552]. From this article it appears that the cdc25 gene has some function in determining cell size. Although there may be equivalent genes and mechanisms in plant genomes, none has yet been identified other than a gene homologous to cdc2. Nor can it be predicted what effect the insertion of the yeast-derived genes into a plant genome by transformation may be.

International Patent Application WO 92/09685 in the name of the Australian National University, published on 11th June 1992, also relates to this subject and reference is directed thereto. The invention described and claimed in that application comprises modulating the level and/or catalytic

activity of a cell cycle protein. The protein identified therein is known as $p34^{cdc2}$ and the level of that protein may be modulated directly by action on the gene itself or indirectly by action taken against one of a number of regulatory genes which regulate $p34^{cdc2}$. Examples of such regulatory genes are given as $p13^{suc1}$, nim-1, wee-1, mik-1 and cdc25. These genes have long been known to exist in yeast but whether they have equivalents in the plant kingdom had remained a matter for speculation. The inventors of WO 92/09685 confirm that $p13^{suc1}$ does have a homologue in plants. From that confirmation the conclusion is drawn that the regulatory genes, nim-1, wee-1, mik-1 and cdc25 from fission yeast will interact with plant $p34^{cdc2}$ and have some controlling effect on plant cell growth. However, this conclusion is based entirely on the known biochemistry of $p34^{cdc2}$ in yeast. No plant transformation has been carried out and there is only suggestion as to the effect that alteration of the cell growth cycle may have at the whole plant level.

An object of the present invention is to provide plants with altered growth characteristics.

According to the present invention, there is provided a method for altering the growth characteristics of plants and plant cells comprising incorporating into the genome of the plant or plant cell by genetic transformation a cdc25 gene or a gene of homologous function.

Preferably the said gene is the cdc25 gene or cdc25 homologue is derived from a yeast, for example, the yeast Schizosaccharomyces pombe.

The said cdc25 gene or cdc25 homologue may be placed under the control of a 35S promoter of Cauliflower Mosaic Virus (CaMV35S).

Alternatively, the said gene may be placed under the control of a chemically inducible promoter enabling expression of the gene to be induced by external application of a chemical inducer.

The invention further provides a recombinant plant genome having stably incorporated within its genome an exogenous cdc25 gene or a functional homologue thereof which, preferably, is derived from a yeast such as Schizosaccharomyces pombe.

The invention also provides genetically modified plants which possess the ability to flower earlier than in the the unmodified form.

Whole plants with altered morphology, reflecting changes at the cellular level, may be produced by regeneration of plants from the said transformed cells. Importantly, the genetic alteration of the genome leads to improvements in the flowering characteristics such as precocious flowering and larger numbers of flowers.

Cells which possess the yeast cdc25 gene display decreased size and plants regenerated from these cells have reduced stature. It may not be considered as entirely surprising that the stature and morphology at the whole plant level is affected by altering cell growth characteristics but what is entirely unexpected and surprising has been our discovery that plants transformed with the cdc25 gene from yeast have a propensity to flower precociously and to produce an increased number of flowers, compared with its wild-type controls, a

fact that is of major significance in crops which are grown for seed.

The inserted gene may be derived from non-plant organisms which possess such genes or equivalents . However, for the purpose of this invention it is preferred that the gene be derived from the fission yeast Schizosaccharomyces pombe. Functional equivalents of the cdc25 gene are those which participate in the control of expression of the p34cdc2 protein, for example wee1, nim1 and suc1.

In our published International Patent Application No. WO 90/08826 (published 9th August 1990) which is incorporated herein by reference, one form of a chemically inducible gene switch is described.

A large number of plant promoters are assumed to be induced using chemical signals. However, it has only been demonstrated in few examples that the specific chemicals switch on gene expression in the tissues required for this invention. The gene of particular interest is the gene encoding the 27kd subunit of glutathione-S-transferase II (GSTII). (See WO 90/08826.) This gene is induced specifically upon treatment of plant tissues using chemical safeners. One such safener is N,N,- diallyl-2,2- dichloroacetamide, but there are related compounds which have improved mobility characteristics in plants tissues, combined with improved persistence for this application, efficacy and safety. These compounds have been described in the literature.

It is obvious that additional chemically induced promoters can be used in this scenario.

Some of these may be of plant origin, others may be of fungal, bacterial or yeast origin. It is implied in the present application that those promoters and chemical combinations suitable for the plant growth control procedure can be used in place of GSTII and safeners.

An additional example is the alcR activator gene and the alcA target promoter from Aspergillus. The chemical inducer is cyclohexanone.

Another example of a chemically inducible gene is given in European Patent Application EP-A-0332104 (Ciba-Geigy).

By placing the cdc25 gene or its homologue under the control of such an inducible gene switch, expression of the gene may be controlled at will by the application of the appropriate chemical inducer to the plant. In the absence of the inducer, the gene does not express and when the inducer is applied the expression is switched on. In the context of this invention, then, the modified plant may be allowed to grow normally until an appropriate stage of its development at which the inducer may be applied and the benefit of the presence of the cdc25 or homologous gene may be obtained. One application is to apply the inducer only at the stage of seed development in order to alter the characteristics of the seed without substantially altering the plant stature or morphology. An alternative method of obtaining such effects is to place the inserted gene under the control of a tissue or organ specific promoter to direct expression to the tissue or organ which is selected for modification, or under control of a development-regulated promoter to restrict

expression to a selected stage in plant development.

The cell division cycle in plants appears to be regulated at key steps by a few genes, although exactly how the gene products interact to regulate cell division is poorly understood. The product of cdc2, $p34^{cdc2}$, plays an important role in both the onset of mitosis and the onset of DNA synthetic (or S-) phase, although there is evidence to indicate that the protein may exist in two forms, one related to S-phase and one to mitosis.

The gene product, $p34^{cdc2}$, is the catalytic sub-unit of the protein kinase MPF (maturation promoting factor), the other two components being the gene products of cdc13 (a "cyclin"). At least three genes interact with the "cdc2 pathway" to regulate the onset of mitosis. The product of cdc25, $p80^{cdc25}$, is required for the dephosphorylation and activation of $p34^{cdc2}$. Wee1 is a negative regulator of the cdc2 protein kinase, delaying entry into mitosis. Nim1 acts to suppress wee1, that is, it is also an activator of mitosis.

Cdc25, wee1 and nim1 are all dose-dependent genes, and therefore even if homologous sequences exist in plants, overexpression of any one of these genes may be expected to alter some aspect of cell division in a plant system.

The invention will now be described, by way of illustration, in the following Examples and with reference to the accompanying drawings.

EXAMPLE 1

A cassette containing cdc25 from Schizosaccharomyces pombe was obtained from Professor Paul Nurse FRS (ICRF Cell Cycle Group,

Unit of Microbiology, Dept. of Biochemistry, University of Oxford, UK). A 1120 base pair fragment containing cdc25 was inserted into two constructs with sense orientation, either with the CaMV constitutive promoter or the endosperm-specific high molecular weight (HMW) glutenin promoter.

The cloning procedure is set out schematically in Figure 1 herewith and details of the constructs used appear in Figures 2 and 3.

Regenerated tobacco plants were obtained following leaf disc transformation using Agrobacterium tumefaciens and the binary vector pBin19. Southern blot analysis confirmed that the regenerated plants did contain the cdc25 construct.

Transformed plants have shown several differences in phenotype compared with the wild-type. In particular, with the constitutive promoter (CaMV35S) the primary transformants are considerably dwarfed, as are the HMW promoter transformants but to a lesser degree.

These results are summarised in Figure 4 which shows in graphical representation the height of the regenerated plants with cdc25 behind the CMV or HMW promoter after 15 weeks.

The second phenotypic change of the CaMV transformants is in leaf character, in particular the leaves appear wrinkled and pocketed, as well as exhibiting altered shape.

The third phenotypic change is an increase (near doubling in some cases) in the number of flowers, achieved through an increase in both the number of flowering branches and the number of flowers per branch. These results are summarised

in Table 1.

TABLE 1

	Average flowers per plant	Average flowers per branch	Average branches per plant
CaMV	49.4	9.2	5.4
HMW	23.1	5.1	3.7

A fourth feature of the transformants was that they flowered early (see data below).

The presence of the CaMV/cdc25 construct does not affect seed viability.

The principal phenotypic change in transformants containing the cdc25 gene under control of the endosperm-specific HMW promoter is the size and viability of the seed, although there is some variability between and within pods. Much of the seed recovered is much smaller than that from the wild type; this is probably because of reduced endosperm size, although the endosperm of tobacco is relatively small. Seed viability varies from 9.4% in some pods to over 90% in pods from the same plant.

Secondary root tips were collected from the dwarf phenotype and control plants and the cell sizes in the root meristems were measured. Figure 5 is a graph showing the relative frequencies of occurrence of a range of cells sizes (plotted as the logarithm).

The results were subjected to statistical analysis. The statistics are summarised in Table 2 and represented graphically in Figures 6 and 7, of which Figure 6 shows the results for control plants and Figure 7 the transformed plants.

TABLE 2

Log Cell Size	Cumulative	Cumulative Frequency: Transformed	Difference Frequency: Control
1.50-1.69	0.093	0.000	0.093
1.70-1.89	0.271	0.052	0.219
1.90-2.09	0.690	0.273	0.417
2.10-2.29	0.930	0.519	0.411
2.30-2.49	1.000	0.818	0.182
2.50-2.69	1.000	0.987	0.013
2.70-2.89	1.000	1.000	0.000

Maximum difference = 0.417

Critical value:

n1 = 129, n2 = 77

at 5% significance level= $1.36 \sqrt{\left(\frac{129 + 77}{129 * 77}\right)} = 0.196$

at 1% significance level= $1.63 \sqrt{\left(\frac{129 + 77}{129 * 77}\right)} = 0.235$

The statistical test used here is the Kolmogorov-Smirnoff two-sample test which is a nonparametric test to show whether two independent samples have been drawn from the same population. What the statistics show is strong evidence that the two distributions are significantly different at the 1% level.

Flowering Characteristics

Seeds of two self-pollinated primary transformants T1S1 containing the constitutively

expressed cassette CaMV-cdc25-nos were germinated and 80 plants potted on. Similarly, 80 plants from a backcross of one of the lines to wild-type (T1BC1) were grown up. There was no selection of germinating seed on kanamycin and, therefore, there should have been a predictable segregation of the T-DNA kanamycin marker in the T1S1 line between homozygotes (KK) and heterozygotes (KO) of 25% KK, 50% KO and 25% OO. The Mendelian segregation would result in a ration of 3:1.

From the outcross between a heterozygous T1BC1 (KO) and (OO) there would be an expected 1:1 ratio of KO:OO of the kanamycin marker in the next generation.

The segregation of kanamycin resistance and the appearance of certain phenotypes were determined. The appearance of particular phenotypes was noted such as the mean number of leaves at flowering. Leaf discs taken from a cross section of the three groups of plants were tested for kanamycin resistance but not all plants were tested. Early and late flowering plants were compared.

At 45 days, 37 out of 40 plants of the T1S1 plants derived from line D and all 40 of line C plants had flowered compared with only 8 of the outcrossed plants.

Of these plants the mean number of leaves to flowering at 45 days was also noted, as follows:

	No. of leaves to flowering 1st	
Line C (CaMV-cdc25-nos)	10.33±0.13	n=40
Line D (CaMV-cdc25-nos)	10.03±0.11	n=37
Line D outcross:		

Precocious flowering phenotype	10.38±0.18	n=8
Normal phenotype	25.75±0.35	n=16

Of the outcross 8 of the 80 plants were flowering at 45 days. Of these 8 plants the mean number of leaves to flowering was very similar to the above two lines C and D; 10.38±0.18, n=8. Of the remaining 72 plants not flowering only 16 were counted to determine the mean leaf number to flowering. There was a significant difference between the former eight and the latter 16 as these plant showed an increase in mean leaf number to flowering to 25.75±0.35, n=16.

Therefore, it would appear that there is a direct correlation between the mean number of leaves and the appearance of early flowering. The flowering appears to be early as N.tabacum cv. Samsun usually flowers at around 80 days and onwards. However, the appearance of early flowering in all of line C and most of line D does not agree with a Mendelian segregation of such a phenotype at 3:1. Neither does a 1:9 ratio of early to non-flowering coincide with the expected 1:1 ratio from an outcross in Mendelian terms. However, the segregation is still of clear importance.

Kanamycin resistance

Leaf discs were taken from surface sterilised leaves with a cork borer and placed on 200μg/ml kanamycin water agar plates. This was carried out at 67 days after potting out of the seedlings. At this point two plants in line D had still not flowered as observed at 45 days.

Of the eight plants identified as precocious

flowering in the outcross, of the two tested, both displayed kanamycin resistance. Of the remainder (exhibiting an increase in average number of leaves to flowering) it was not possible to determine kanamycin resistance because of fungal contamination of the leaf discs despite surface sterilisation and aseptic plating techniques.

Of the 10 plants tested from both of lines C and D, of those that survived, in line D six were found to be resistant to kanamycin and eight in line C.

There appears to be a link between the presence of kanamycin resistance and the reduction of the average number of leaves to flowering. The appearance of other phenotypes associated with the T1 lines were observed in the T2 generation but no correlation was made between their appearance and the kanamycin resistance marker.

Discussion

It has already been demonstrated that when the seed of lines C and D are germinated on kanamycin plates there is a clear segregation of those seeds into bleached (kanamycin sensitive) and green (kanamycin resistant) seedlings in a ration approximating to 1:4. This is close to the 1:3 ratio expected. This however does not correlate with the kanamycin resistance observed in the plants using the leaf disc tests. It is believed that this is caused by the low sample numbers available for testing.

CLAIMS

1. A method for altering the growth characteristics of plants and plant cells comprising incorporating into the genome of the plant by genetic transformation a cdc25 gene or a gene of homologous function.

2. A method as claimed in claim 1 in which the said gene is the cdc25 gene or cdc25 homologue is derived from a yeast.

3. A method as claimed in claim 1, in which the yeast is Schizosaccharomyces pombe.

4. A method as claimed in claim 1, in which the said cdc25 gene is under the control of a 35S promoter of Cauliflower Mosaic Virus (CaMV35S).

5. A method as claimed in claim 1, in which the said cdc25 gene is under the control of a tissue- or organ specific promoter.

6. A method as claimed in claim 1, in which the said cdc25 gene is under the control of a developmentally-regulated promoter.

7. A method as claimed in claim 1 in which the said gene is under the control of a chemically inducible promoter enabling expression of the gene to be induced by external application of a chemical inducer.

8. A recombinant plant genome having stably incorporated within its genome an exogenous cdc25 gene or a functional homologue thereof.

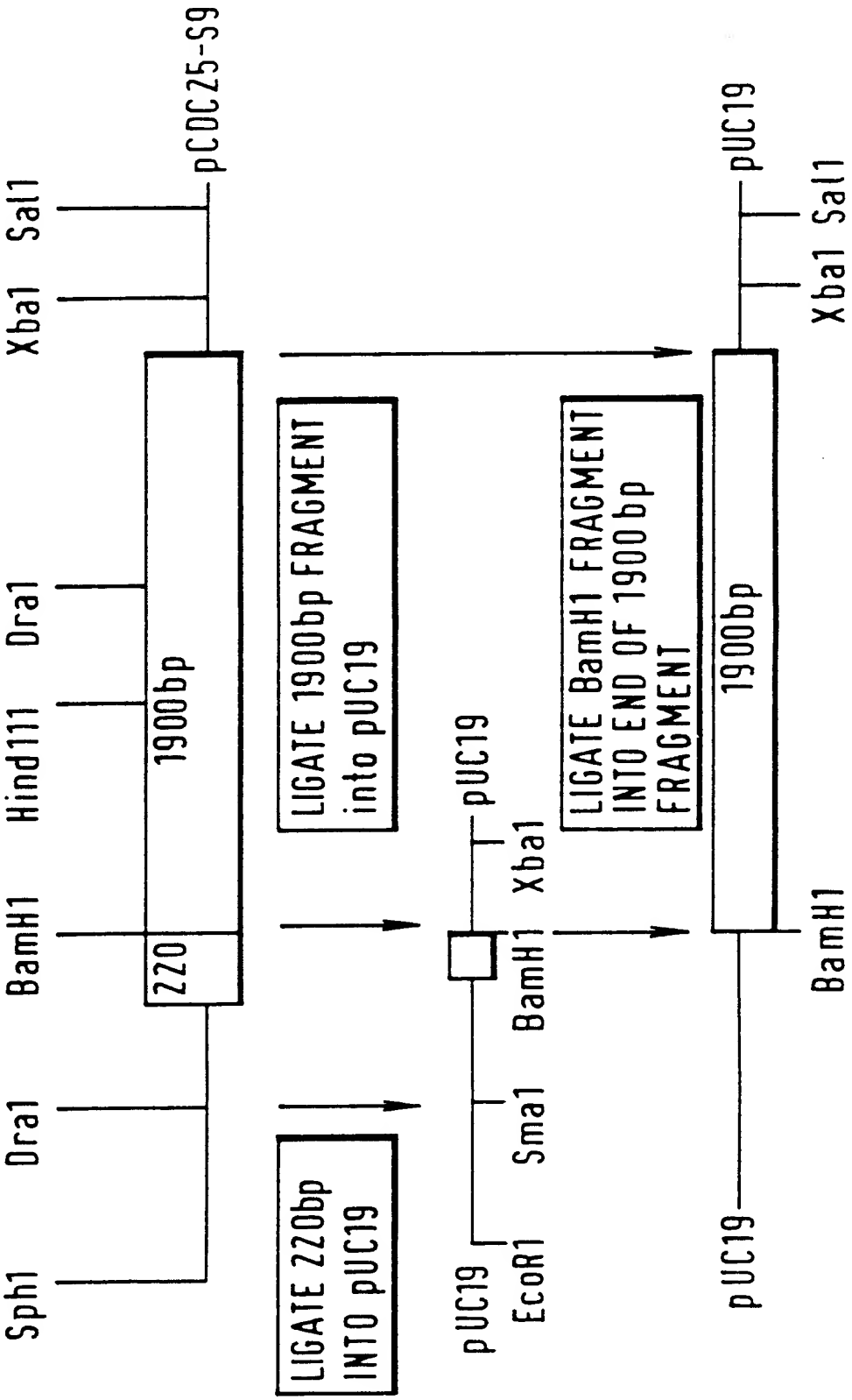
9. A recombinant plant genome as claimed in claim 8, in which the said cdc25 gene or functional homologue thereof is derived from a yeast.

10. A recombinant plant genome as claimed in claim 9, in which the yeast is Schizosaccharomyces pombe.

11. A genetically modified plant having the ability to flower earlier than in the the unmodified form.

12. An early flowering genetically modified plant, as claimed in claim 11, having a genome as claimed in claim 8 or claim 9 or claim 10.

FIG. 1
STRATEGY USED TO CLONE cdc25 INTO pUC19



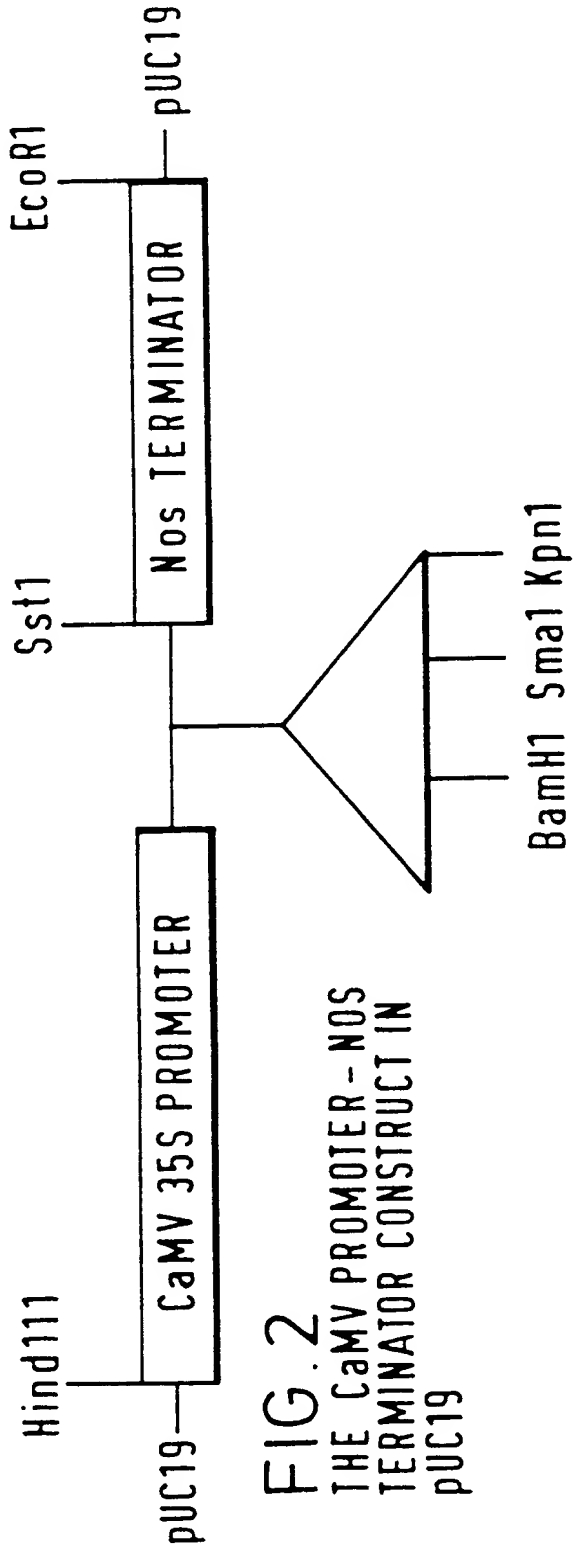


FIG. 2
THE CaMV PROMOTER-NOS TERMINATOR CONSTRUCT IN pUC19

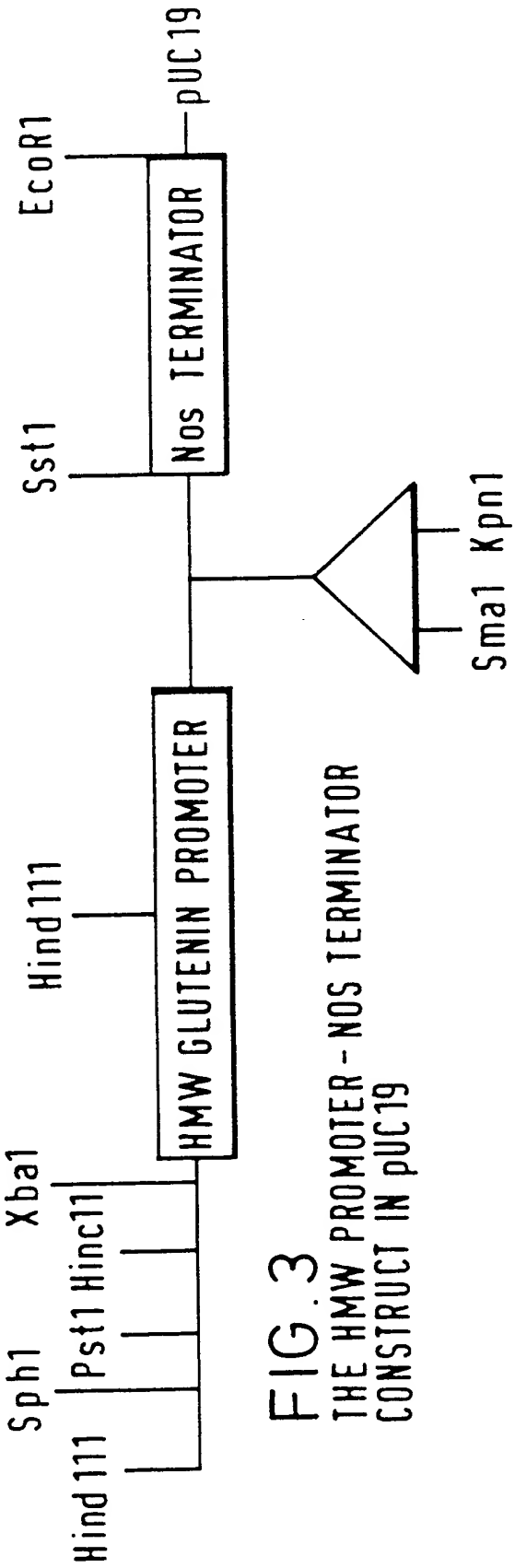


FIG. 3
THE HMW PROMOTER-NOS TERMINATOR CONSTRUCT IN pUC19

FIG. 4

HEIGHTS OF REGENERATED PLANTS WITH cdc25 BEHIND THE CMV OR HMW PROMOTER AFTER 15 WEEKS

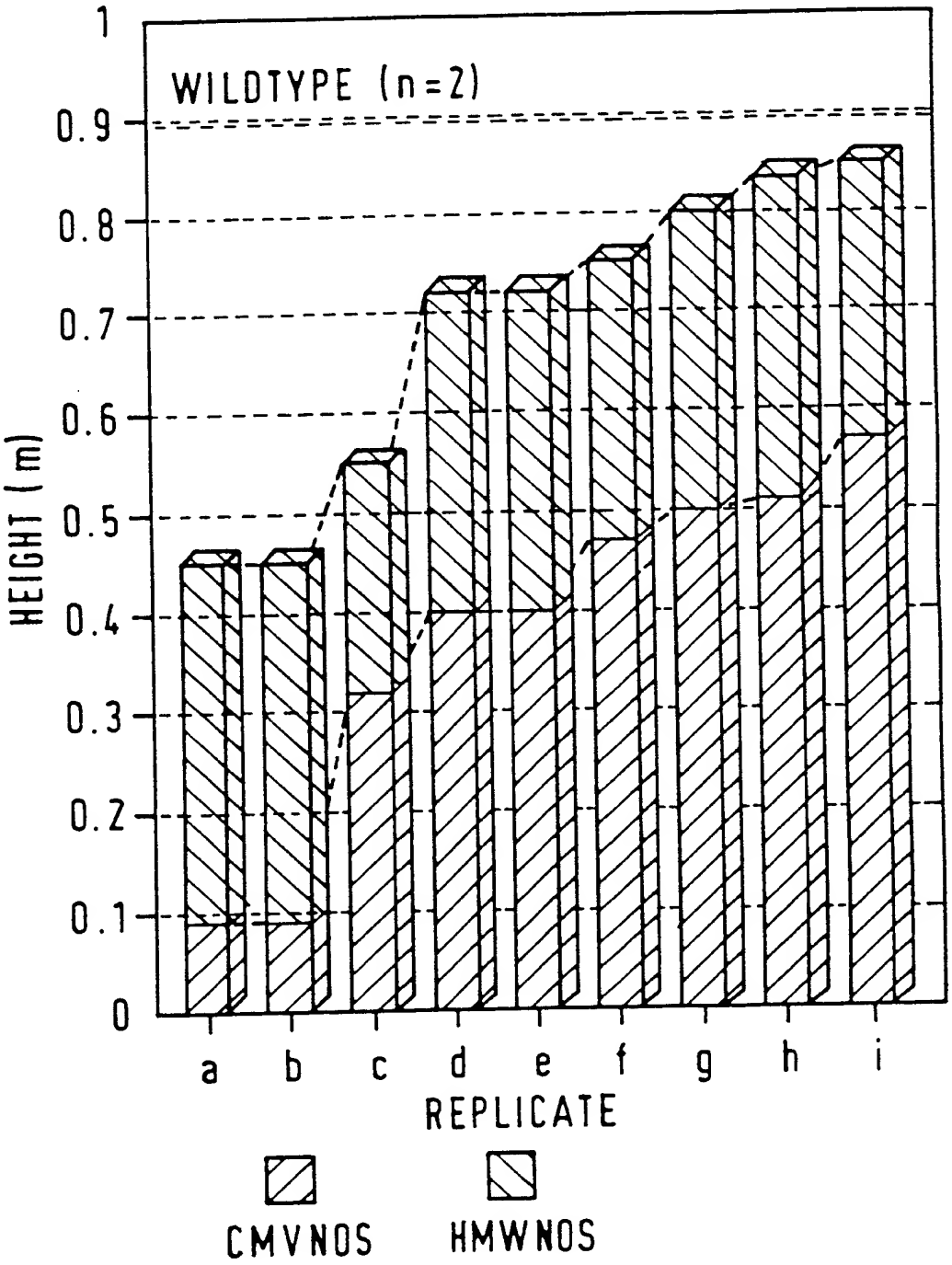


FIG. 5

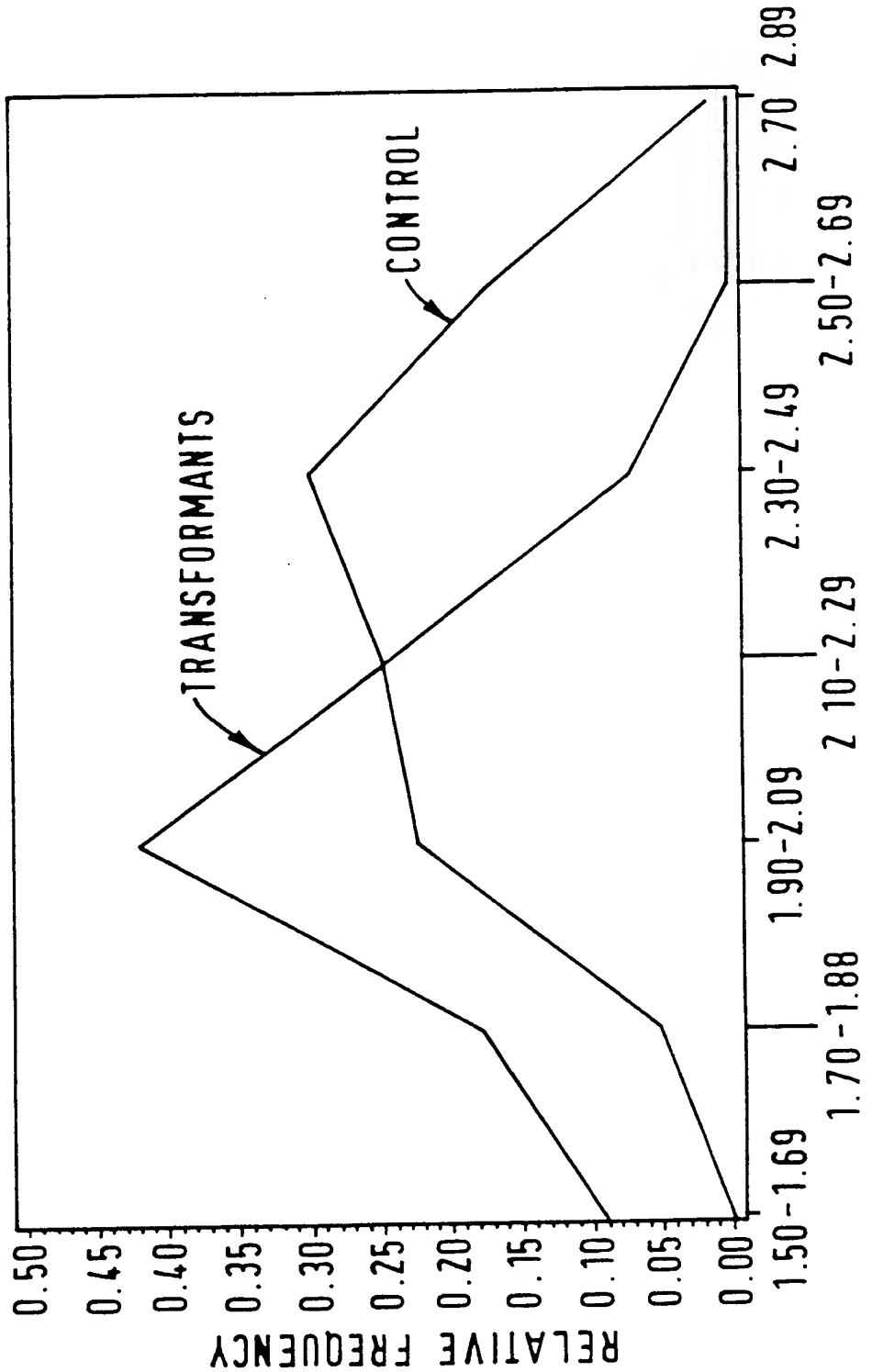
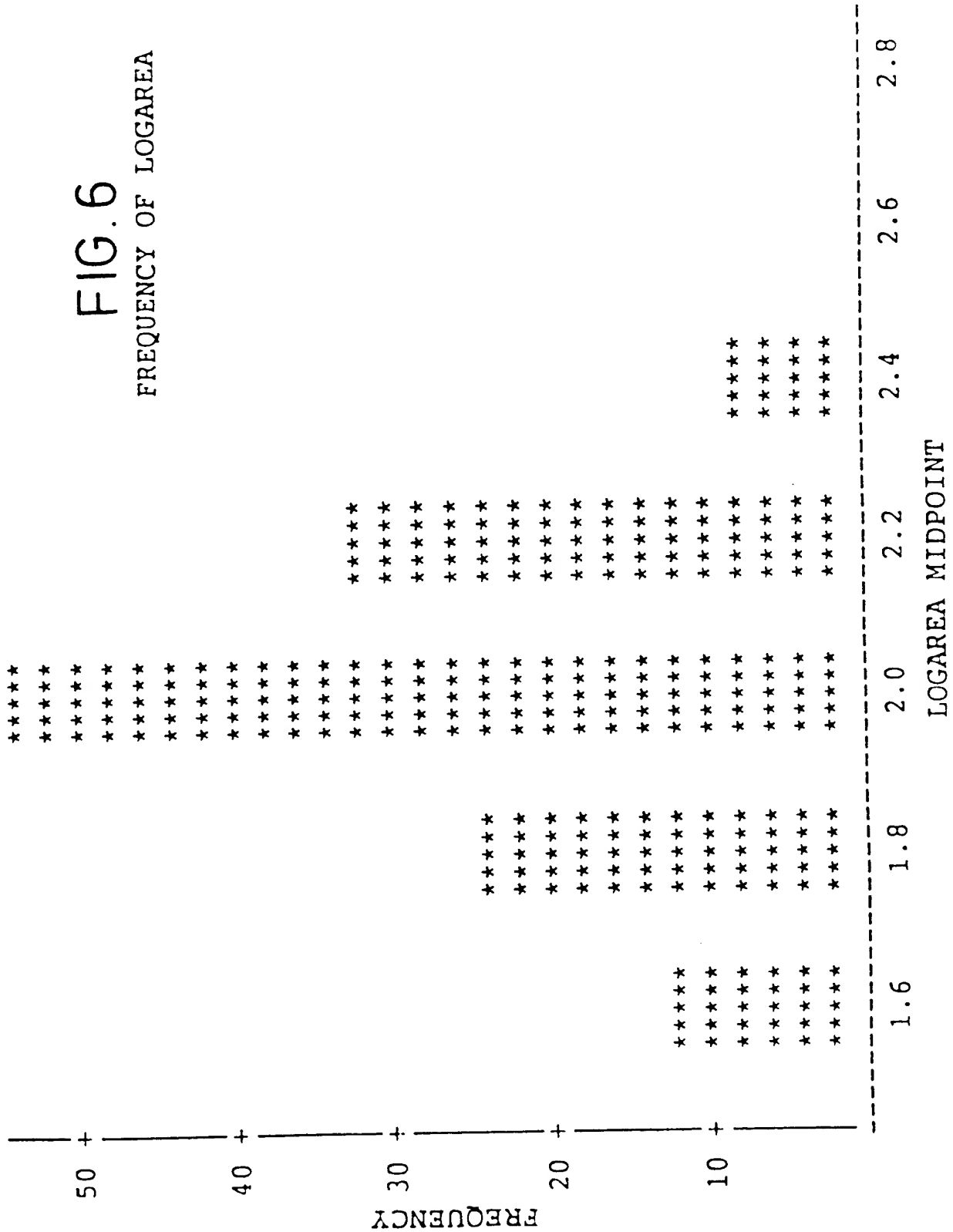

RELATIVE FREQUENCY
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
1.50-1.69
1.70-1.88
1.90-2.09
2 10-2.29
2.30-2.49
2.50-2.69
2.70 2.89
TRANSFORMANTS
CONTROL

FIG. 6


FREQUENCY OF LOGAREA
FREQUENCY
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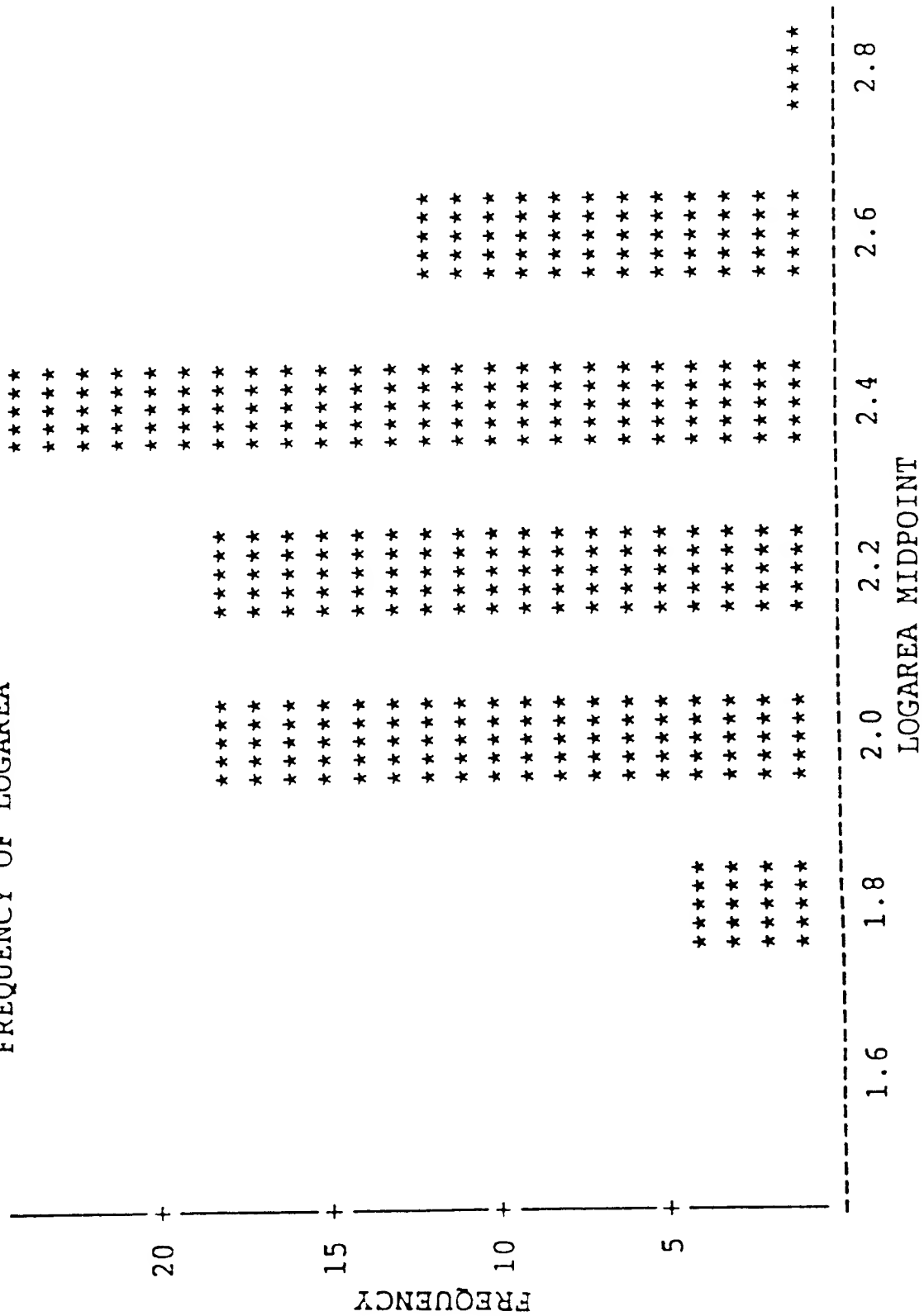
2.8
LOGAREA MIDPOINT

FIG. 7


FREQUENCY OF LOGAREA
FREQUENCY
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1.6
1.8
2.0
2.2
2.4
2.6
2.8
LOGAREA MIDPOINT

INTERNATIONAL SEARCH REPORT

International Application PCT/GB 92/02340

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N15/31; C12N5/10; C12N5/04
A01H4/00; A01N63/00; A01H5/02

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H ; A01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	BIOLOGICAL ABSTRACTS vol. 90 , 1990, Philadelphia, PA, US; abstract no. 15284, OONO, Y., ET AL. 'Early flowering in transgenic tobacco plants possessing the rolC gene of Agrobacterium-rhizogenes Ri plasmid' see abstract & JPN. J. GENET. vol. 65, no. 1, 1990, pages 7 - 16	11,12
P,X	WO,A,9 209 685 (AUSTRALIAN NATIONAL UNIVERSITY) 11 June 1992 cited in the application see page 8, line 19 - line 32; claims 18-22,33-36	1-10

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
24 MARCH 1993	06.04.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	PLANT MOLECULAR BIOLOGY. vol. 14, 1990, DORDRECHT, THE NETHERLANDS. pages 669 - 685 HILSON, P., ET AL. 'Yeast RAS2 affects cell viability, mitotic division and transient gene expression in Nicotiana species' see the whole document ---	1-12
A	CELL vol. 45, 11 April 1986, CAMBRIDGE, MA US pages 145 - 153 RUSSELL, P., ET AL. 'cdc25+ functions as an inducer in the mitotic control of fission yeast' see the whole document -----	1-12

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9202340
SA 67849

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9209685	11-06-92	None	

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82